The Role of Protein Kinase C Isozymes on the Zebrafish HSC Microenvironment

Research Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in the undergraduate colleges of The Ohio State University

by

William Deruelle

The Ohio State University
December 2020

Project Advisor: Dr. Bradley Blaser, Department of Internal Medicine, College of Medicine

Second Project Advisor: Dr. Mark Ziolo, Department of Physiology and Cell Biology, College of Medicine

Abstract

Long-lived hematopoietic stem cells (HSCs) are cells that self-renew and generate all mature blood cell types.[1] HSCs reside in a microenvironment composed of multiple distinct cellular niches that provide critical signals for adhesion, survival, quiescence and proliferation.[2] The mechanisms by which these niches regulate the long-term fate of HSCs are incompletely known but are critical to our understanding of leukemogenesis and reconstitution of hematopoiesis after hematopoietic stem cell transplantation (HSCT). Prior studies have shown that the endothelial cell niche of the developing zebrafish can be modulated by overexpression of the angiogenic chemokine receptor, *cxcr1*, to increase its capacity to support hematopoietic stem and progenitor cells (HSPCs) in short term assays.[3] In preliminary data from the Blaser Laboratory, 219 candidate genes with differential expression between niche- and non-niche endothelial cells were identified. A gain of function screen identified the zebrafish protein kinase C (PKC) isoform *prkcda* (PKC delta a) as a positive regulator of short- and long-term HSC fate. *Prkcda* is a member of related proteins that are important second messengers for extracellular communication signals. I hypothesized that PKC isozymes have specific roles in regulating HSC fate by affecting the function of the endothelial cell niche. RNA sequencing showed no significant differences in expression of any of these isozymes in niche endothelial cells compared with non-niche endothelial cells. To functionally test the role of each, I generated zebrafish with transgenic expression of the isozyme in the niche and found no significant differences in HSC numbers. To begin to understand the requirement for each isozyme, I made loss of function mutants using CRISPR/Cas9 which will be studied more in the future. Elucidation of the role of PKC signaling within the hematopoietic microenvironment may enhance our understanding of leukemogenesis and hematopoietic reconstitution after HSCT.

Introduction

PKCs are serine and threonine kinases capable of regulating cell proliferation, gene expression, migration, and apoptosis.[4] Twelve PKC isozymes are known,[5] each categorized based on their structure and unique substrate targeting preferences.[6] Three PKC subgroups are categorized by their unique activation signals: conventional PKC (α, βI, βII, and γ) respond to diacylglycerol (DAG), phosphatidylserine (PS), and Ca^{2+}; novel PKC (δ, ε, η, θ, and μ) respond to DAG and PS; and atypical PKC (ζ, λ, and ι) respond to neither DAG nor Ca^{2+} but require PS.[5-7] Abnormal PKC expression in humans has been implicated in cancer and heart disease.[5,8]

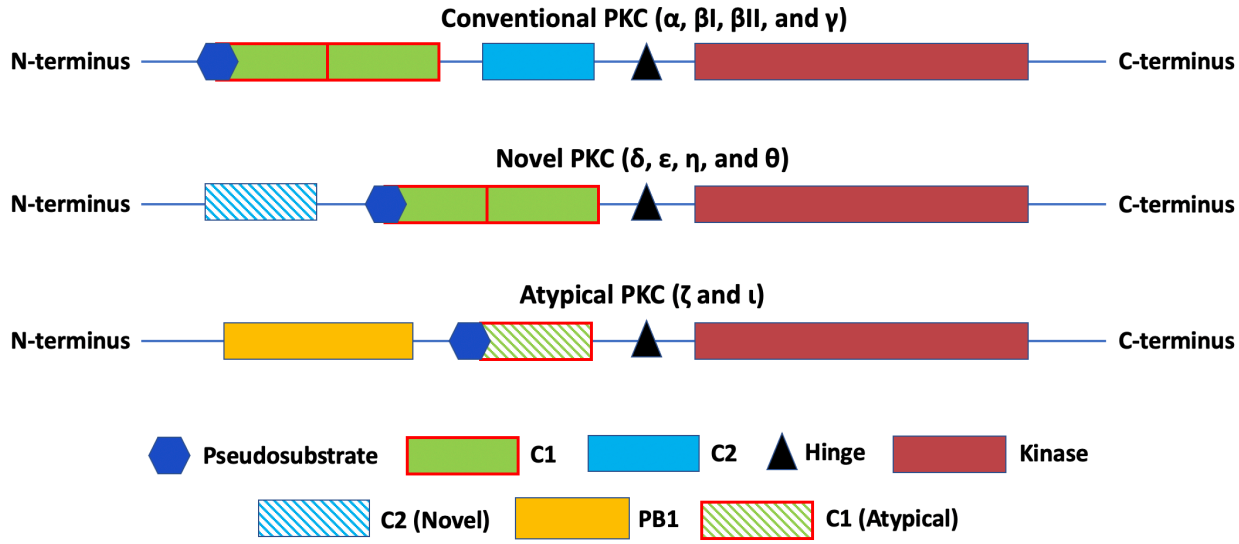


Figure 1. Three PKC subgroups containing conserved, PB1, and kinase domains.

The PKC structure includes a regulatory amino-terminus and a catalytic carboxy-terminus (Figure 1).[9] Way et al[6] noted that PKC isoforms vary in structure predominantly in the conserved region. Conventional PKCs contain four conserved and five variable regions.[10] Two of the conserved domains, C1 and C2, are regulatory and interact with DAG, PS, and Ca^{2+} for binding to the lipid bilayer of the cell.[6] The sole C1 region of atypical PKCs does not bind DAG due to the basicity of its binding residues.[11,12] Unlike conventional PKCs, the C2-like region of novel PKCs

does not bind Ca^{2+}.[13] The catalytic C3 region binds ATP, and the C4 region, also catalytic, is involved in the phosphorylation of substrates.[6] Both C3 and C4 are part of the conserved kinase domain in the conventional, novel, and atypical PKC subgroups.[14] Cellular processes associated with PKC activation include signaling via the G-protein coupled receptor (GPCR) second messenger cascade, extracellular ligand binding, communication with different PKCs, and other molecular binding or signaling.[15-18]

In conventional PKC signaling, phospholipase C (PLC) and G-protein coupling are initiated upon first messenger binding to the GPCR.[19] One example of first messenger signaling includes the *cxcl8* chemokine and associated *cxcr1* and *cxcr2* receptors. The G-protein associated receptors bind the chemokine and activate the signaling cascade via PLC.[19] *Cxcl8* and *cxcr1* expression in zebrafish endothelial cells has been shown to enhance HSPC colonization of the caudal hematopoietic tissue (CHT).[3] Likewise, *cxcr4* can bind the chemokine ligand *cxcl12*, activating a cascade through PLC.[20] *Cxcr4* expression occurs in HSCs and endothelial cells,[21] and the *cxcl12/cxcr4* signaling pathway has been associated with cell adhesion, cell proliferation and cancer metastasis.[22] The chemokine receptors and related signaling cascades are potential targets for cancer treatments,[23] which can inhibit PKC activation.[17]

Activated PLC separates phosphatidylinositol 4,5-bisphosphate (PIP_2) into second messengers, DAG and inositol trisphosphate (IP_3).[24] IP_3 will then enable Ca^{2+} ion release from the endoplasmic reticulum.[25] Ca^{2+} binds the regulatory C2 region, attracting PKC to the anionic PS of the phospholipid bilayer.[26] PKC's C1B region binds DAG and PS at the membrane, thereby converting the autoinhibited PKC into an active enzyme following the removal of a pseudosubstrate from the kinase.[27] The conventional PKC is then able to phosphorylate proteins and conduct further signaling within the cell.[27]

Unlike conventional and novel PKCs, atypical PKCs utilize a PB1 region in order to bind protein scaffolds.[28] Whereas conventional PKC isozymes bind DAG prior to pseudosubstrate removal from the kinase, protein scaffolds containing a PB1 domain bind the atypical PKC PB1 domain to activate the kinase.[28] Examples of protein scaffolds include p62 and Par6,[28] which enable the PKC's open confirmation.[29] Placement of the PKC and corresponding substrate on the scaffold improves phosphorylation since atypical PKCs maintain slow reaction rates.[11]

Disturbances of PKC isoform signaling cascades can have implications for leukemogenesis. According to Nakagawa et al,[30] a PKCα dominant-negative mutation in hematopoietic progenitor cells (HPC) of a murine fetal liver resulted in B lymphocytes transformation, resembling phenotypic B cells found in B-Cell Chronic Lymphomatic Leukemia (B-CLL). Dominant negative PKCα plasmids were introduced to the HPCs via a retrovirus and compared with MIEV control cells. PKCα mutation caused the proliferation of these HPCs, which was 1.5x greater than that of the control, during the beginning stages of B-cell transformation. Notably, B cells expressing the mutation survived without IL-7 growth and OP9 stromal factors while 3x more control cells underwent apoptosis under similar conditions. These findings indicate a unique role for PKCα in the transformation of B-cells.[30]

PKCδ (*prkcda/b* in zebrafish) supports cellular apoptosis under certain conditions,[31] and this isozyme may be downregulated in some forms of cancer.[32] Apoptosis of cells is associated with the phosphorylation of tyrosine (Tyr), which activates PKCδ.[31] Phosphorylation of Tyr-64 and Tyr-155,[33] as well as Tyr-311 and Tyr-332,[34] residues in PKCδ are associated with apoptosis. Chemotherapy is known to cause PKCδ translocation to the nucleus and disrupting this translocation has resulted in the inhibition of cellular apoptosis caused by etoposide.[33] The phosphorylation sites related to apoptosis in etoposide treatments are Tyr-187 and Tyr-64.[33] Tyr-

155, Tyr-187, and Tyr-64 are located in the regulatory region,[33] and Tyr-311 lies within the hinge domain, which is located between PKCδ's catalytic and regulatory regions.[34] Additionally, chromosome 3p, which houses the PKCδ gene, is associated with tumor suppression.[35]

PKCε is linked to cell survival and proliferation.[36] Cell proliferation is regulated by the RAF1 kinase, which begins the signal cascade involving MEK and ERK phosphorylation.[37] Overexpression of the PKCε isoform in HSCs has been shown to offer protection against apoptosis.[36] PKCε overexpression has resisted etoposide-induced apoptosis in Non-Small Cell Lung Cancer (NSCLC) cells.[38] Increased expression of the PKCε isoform has been discovered in both breast[37] and prostate cancers.[40] Murine studies involving prostate-specific lines showed that contrary to PKCα and PKCδ expression where the prostate was normal, the experimental group experienced preneoplastic lesions in the prostate upon elevated PKCε expression.[41]

PKCλ/ι is an atypical PKC that can serve to promote or suppress tumors, depending on the microenvironment.[42] PKCλ/ι has been linked to tumor initiation in human Lung Adenocarcinoma lines in which PKCλ/ι phosphorylates ELF3, resulting in NOTCH3 expression. Inhibition of this signaling pathway was found to prevent tumor formation in vivo.[43] Furthermore, Justilien et al showed that by preventing Ect2 expression in the PKCλ/ι-Ect2-Rac1-NPM signaling pathway, tumor formation in murine lungs could be inhibited.[44] Later in cancer development and depending on the microenvironment context, however, PKCλ/ι expression may support tumor suppression.[42] The isoform's underexpression in this case can lead to proliferation and tumorigenesis.[42]

The whole genome duplication in zebrafish has resulted in prkc gene paralogs, including *prkcaa/b* (PKC alpha a/b) and *prkcba/b* (PKC beta a/b), requiring analysis of differing expression patterns.[45] Preliminary studies performed by the Blaser Lab have shown that prkc isozyme impacts

on HSPC engraftment within the endothelial cell niche can be ascertained using the zebrafish model. Zebrafish embryos were generated carrying two transgenic reporter genes: *kdrl:GFP* marking all endothelial cells and *lyve1b:DsRed*, marking the endothelial cells of the hematopoietic niche (Caudal Hematopoietic Territory, CHT, Figure 2). Double transgenic embryos were dissociated, and niche and non-niche endothelial cells were isolated by FACS. Bulk RNA sequencing was performed on the sorted cells and 219 differentially regulated genes were identified between the two cell types (Figure 3).

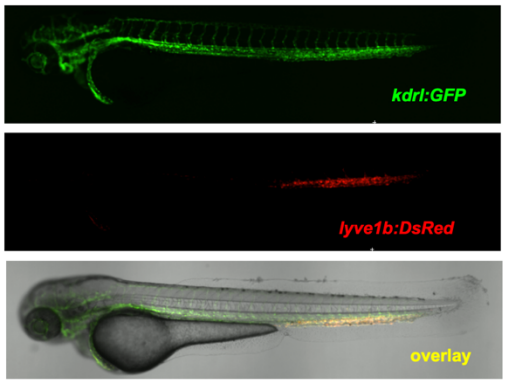


Figure 2. Gene expression in non-niche and niche endothelial cells.

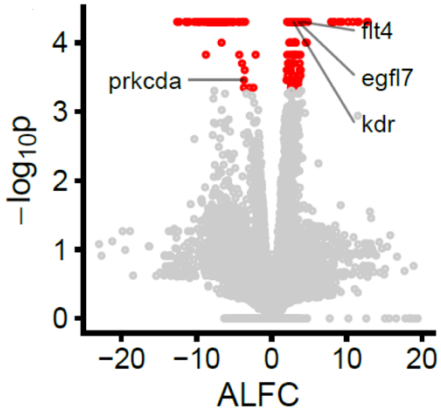


Figure 3. Flow cytometry and bulk RNA sequencing of differentially regulated genes.

A gain of function screen was performed by expressing candidate differentially regulated genes within the hematopoietic niche using a niche-specific enhancer element identified upstream of the E selectin gene (*sele*). Expression constructs were injected into *Runx1:GFP* embryos at the single cell stage. These fish carry an HSPC-specific fluorescent reporter transgene. Microinjection of an empty vector into clutchmate embryos served as a control. In both experimental and control groups, expression of the microinjected plasmid was identified by coexpression of a fluorescent *mCherry* reporter. Compared to the *sele:mcs-2A-mCherry* control injections, animals with a *sele:prkcda-2A-mCherry* construct had significantly more HSCs within

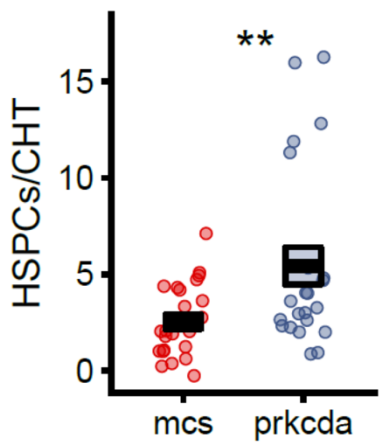


Figure 4. HSPC quantification within CHT.

the CHT (5.4 ± 1.0 vs 2.5 ± 0.4, p = 0.01, Figure 4). In order to understand the effects on long-term HSC fate and clonal diversity, genetically barcoded GESTALT embryos were microinjected with the *sele:prkcda-2A-mCherry* or control plasmids. Beginning at 3 months post-fertilization, peripheral blood was sampled, and genetic barcodes were amplified and sequenced to determine the number of phylogenetically distinct HSCs contributing to hematopoiesis. Animals injected with the *sele:prkcda-2A-mCherry* expression constructs had significantly greater phylogenetic diversity compared to control animals from 6 to 12 months post-fertilization (Figure 5). These results suggested that the increased HSCs resulting from *prkcda* overexpression in the CHT led to a greater clonal diversity throughout life. The overall aim of this thesis project is to better understand the role of other PKC isoforms in controlling HSC clonal diversity via expression in the CHT.

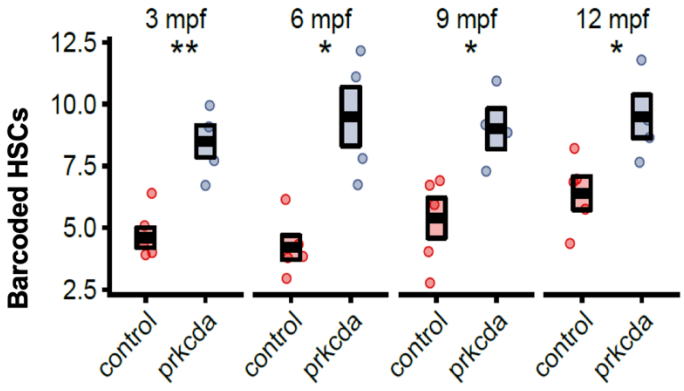


Figure 5. Dysregulated niche expression of *prkcda* increases HSC clonal diversity

Materials and Methods

Animals

Runx1:GFP, GESTALT, and *casper* zebrafish were kind gifts of Leonard Zon. Animals were housed in the OSUCCC Aquatic Facility in automated recirculating racks (Tecniplast) using

standard husbandry practices (Westerfield, The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Danio rerio*)). All experiments were performed under IACUC protocol 2018A00000012.

Cloning

Coding sequences for PKC isoforms were identified in Ensembl (http://www.ensembl.org). Primers were designed to amplify the primary transcript according to APPRIS criteria, adding a consensus Kozack sequence (CACC) prior to the first codon and removing the stop codon. Total RNA was harvested from 72 hpf zebrafish embryos and cDNA made by reverse transcription (Superscript IV, Invitrogen). PKC coding sequences were amplified and cloned by Topo reaction into SD/D Topo Gateway-compatible middle entry vectors (Invitrogen). LR Clonase reactions were performed, according to the manufacturer's instructions, at 0.5X scale using p5e-sele-792 (5' entry), p3e-2A-mCherry (3' entry) and 394 (Tol2 compatible backbone) plasmids. Final expression constructs were screened by restriction digest and confirmed by whole plasmid sequencing (MGH DNA Core).

Embryo microinjection

Tol2-based expression constructs were used for all transgenesis experiments. Tol2 is a zebrafish retrotransposon element and all expression constructs carry LTR integration sequences flanking the regions of interest. Tol2 mRNA was synthesized (mMessage mMachine) and coinjected with expression constructs to facilitate integration (100 pg per injection). Injection mixes were generated in order to deliver 20 pg plasmid DNA in 1 nL injection volume. Following microinjection, embryos were incubated at 28 °C.

For CRISPR/Cas9 injections, high-efficiency sgRNA sequences were identified using ChopChop (https://chopchop.cbu.uib.no/). Commercially-prepared sgRNA CRISPR guides

targeting *prkcba*, *prkcea/b*, *prkcda/b*, *prkcg*, and *prkcha* (Horizon Discovery), were injected with recombinant Cas9 enzyme (New England Biolabs) into the mutant casper zebrafish line.

Microscopy and Image analysis

At 72 hpf, embryos were anesthetized with tricaine, mounted in agarose and imaged on a Keyence BZX-700 digital fluorescence microscope. Images were obtained at 10X magnification using 2-micron Z-slices, acquiring 3 Z stacks per animal to cover the entire CHT. Images were stitched and maximum intensity projections were generated using Keyence software. Images were analyzed in ImageJ. Each image was scored in a blinded fashion and confirmed by an independent evaluator.

Mutation genotyping

To confirm CRISPR guide mutation frequency in F_0 CRISPR mutants, zebrafish embryos were pooled, and genomic DNA purified using the Zymo DNA prep kit. 200 to 300 bp amplicons were generated using flanking primers and submitted for next generation sequencing. Sequencing results were analyzed using a custom informatics pipeline. Adult F_1 generation zebrafish were genotyped by finclip; PCR products were generated with sample indices, pooled, sequenced and analyzed in a similar fashion.

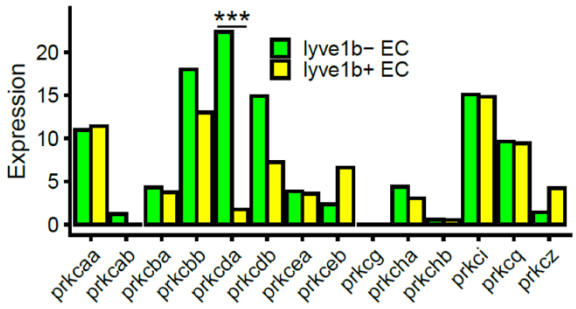


Figure 6. Differential expression of PKC in non-niche and niche endothelial cells.

Results

Expression of PKC isoforms in niche-derived and non-niche-derived endothelial cells.

RNA sequencing data from *kdrl:GFP*$^{+}$*;lyve1:DsRed*$^{+}$ niche-derived endothelial cells and *kdrl:GFP*$^{+}$*;lyve1:DsRed*$^{-}$ non-niche-derived endothelial cells was queried to identify expression

of all known PKC isoforms. Only *prkcda* showed significantly differential expression between these cell types (Figure 6)

Anatomic expression pattern of pkcda in 72 hpf zebrafish embryos

Whole mount *in situ* hybridization was performed to determine the expression pattern of *prkcda* in zebrafish embryos at 72 hpf. This showed expression in the heart and otic vesicles. No expression was detected in the CHT (Figure 7).

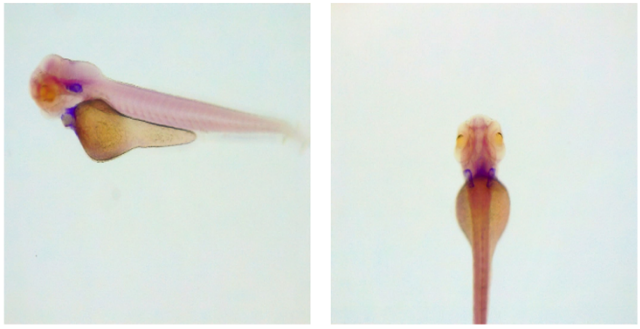
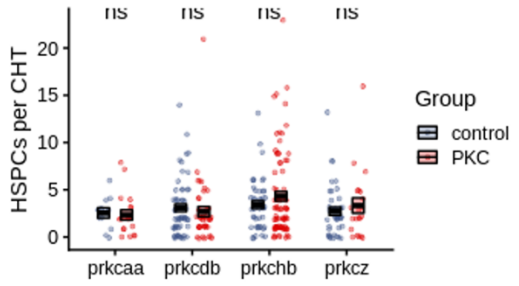


Figure 7. *Prkcda* expression in heart and otic vesicle *72 hpf*.

Gain of function testing

Functional testing was performed using *sele:PKC* expression constructs. A total of 315 *Runx1:GFP* animals were microinjected with these constructs or empty vector control constructs and imaged at 72 hpf. The number of HSPCs within the endothelial cell niche was quantified. The genes tested included *prkcaa*, *prkcdb*, *prkchb*, and *prkcz*, corresponding to PKC alpha a, PKC delta b, PKC eta b, and PKC zeta. No significant difference in HSPC numbers was found between any of the PKC groups and their corresponding controls (Figure 8).



Gene	Category	Fish Imaged
prkcaa	control	11
	sele:prkcaa	18
prkcdb	control	57
	sele:prkcdb	42
prkchb	control	45
	sele:prkchb	81
prkcz	control	40
	sele:prkcz	21

Figure 8. HSPC quantification of *prkcaa*, *prkcdb*, *prkchb*, and *prkcz*.

Prkcda CRISPR mutants

CRISPR mutants were made by selecting guide RNAs with high predicted cutting efficiency for *prkcda* using ChopChop (https://chopchop.cbu.uib.no/). Guide RNAs were injected with recombinant Cas9 protein, and a sample of F_0 embryos were pooled and typed by next generation sequencing. Sequencing data was analyzed using a custom pipeline to identify frameshift indel mutations. Mutagenesis frequency was approximately 65% for frameshift indels (Figure 9). These

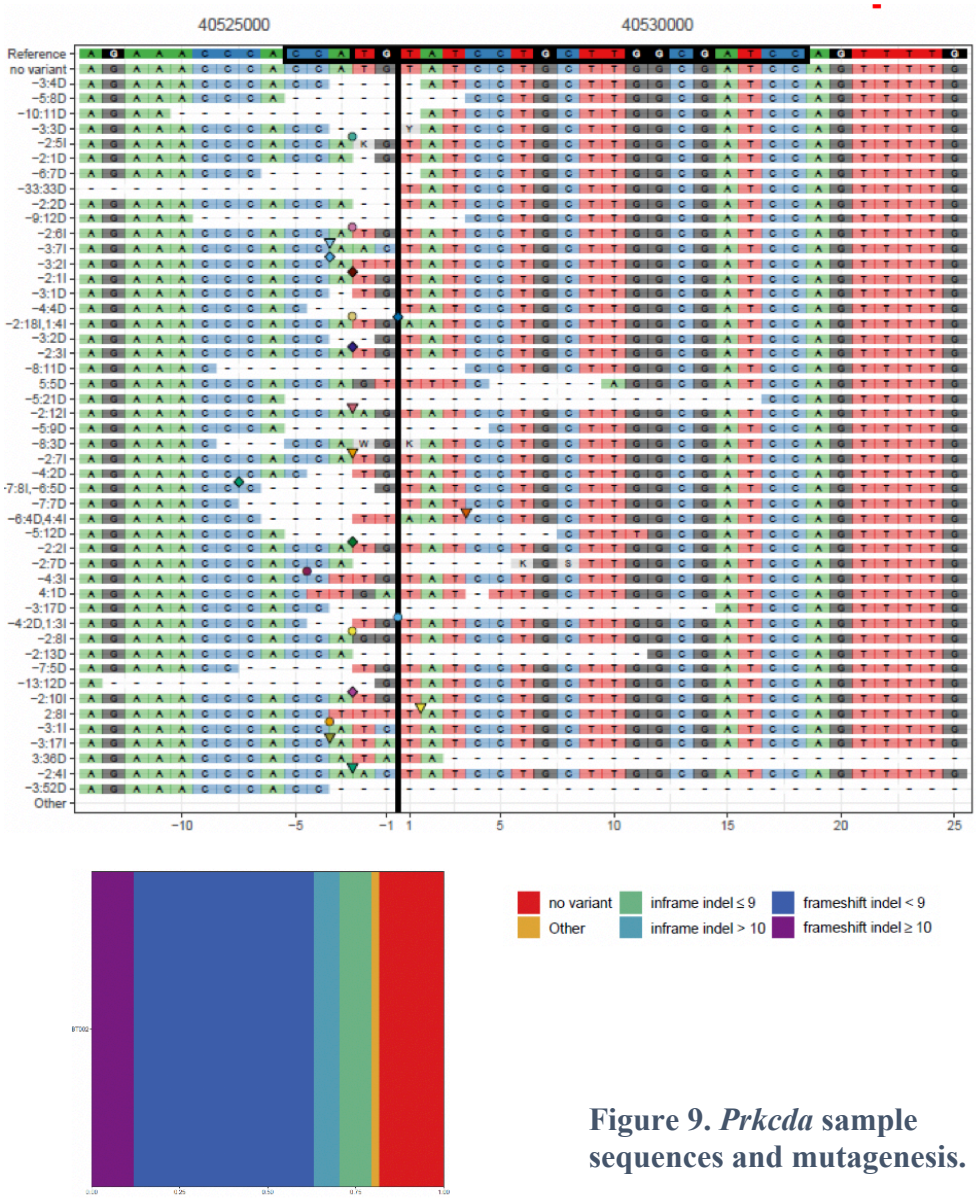


Figure 9. *Prkcda* sample sequences and mutagenesis.

animals have been raised to adulthood and are being screened for germline transmission of frameshift alleles.

Discussion

In an unperturbed state, *prkcda* exhibits low levels of expression in niche endothelial cells and high levels of expression in non-niche endothelial cells. Overexpression of *prkcda* in the CHT, however, resulted in an increased number of HSCs within this microenvironment. Although a specific mechanism for *prkcda* signaling has yet to be uncovered, this modulation of HSCs within the niche suggests that *prkcda* has a regulatory role following its introduction into the microenvironment. This revised role of *prkcda* upon overexpression may be the result of PKC crosstalk upon a change in microenvironment context, leading to altered downstream signaling of *prkcda* itself or among other PKC isoforms normally residing in the endothelial cell niche. For example, Kim et al[16] reported that mitogen-activated protein kinase (MAPK) modulation in cells containing PKCζ overexpression relied on conventional and novel PKC isoform expression as PKCζ is not responsive to DAG analogues. Therefore, it is possible that there is an unidentified synergistic effect among PKC isozymes within the endothelial cell niche.

The *cxcl8/cxcr1/prkcda* signaling pathway may support increased HSC numbers via endothelial niche remodeling. Our lab has shown that human umbilical vein endothelial cell treatment with recombinant *cxcl8* chemokines in vitro resulted in increased accumulation of PKC-protein. Enforced expression of *cxcl8* by HSPCs in Runx1+23:cxcl8 zebrafish embryos increased stem cell numbers.[46] These results support the premise that PKC isoforms may function to enhance blood cell development only upon appropriate cytokine binding at the GPCR.

The increase in endothelial-cell niche HSCs resulting from *prkcda* overexpression is intriguing given the isozyme's common role in apoptotic pathways. Recent experiments by the Blaser lab suggest that *cxcl8/cxcr1* signaling within the niche may supplant apoptotic pathways involving phosphorylation of the kinase's regulatory tyrosines, such as Tyr-187 and Tyr-64. The upstream chemokine signaling and subsequent downstream kinase activity influencing stem cell production is perhaps due to the change in microenvironment context experienced by *prkcda* upon enforced expression in the CHT.

Cxcl8 is associated with cell survival and signals through both the phosphoinositide-3-kinase (PI3K) and MAPK signaling cascades.[47] A study by Grossoni et al[48] noted that PKCδ overexpression in murine NMuMG mammary cells led to cell proliferation and MAPK-ERK activation. Importantly, the study's authors highlighted that this isoform's overexpression resulted in resistance to chemotherapy treatments. Instead, stimuli associated with apoptosis had the unexpected effect of increasing cell proliferation. PKCδ expression has a dual role of apoptosis and cell proliferation, depending on unique signaling within the microenvironment context.[48] The *cxcl8/cxcr1/prkcda* pathway's stimulation of endothelial cell niche expansion and increased HSPC cuddling in the Blaser lab experiments correlates with the findings by Grossoni and others that pertain to the gene's role of positive regulation in certain contexts. The role of this pathway in leukemogenesis is currently unclear, however.

Based on 72 hpf quantification, enforced expression of other PKC genes (*prkcaa*, *prkcdb*, *prkchb*, and *prkcz*) within the niche did not cause a significant change in HSC numbers. Initial data indicates a lack of significant differential expression among non-niche and niche endothelial cells. The exact role of these PKC isozymes within the zebrafish CHT and their influence on HSC populations remains to be discovered. The fact that *prkcdb* expression did not resemble

that of its paralog, *prkcda*, is interesting and examination of the mutational differences between the two isoforms may further our understanding of *prkcda's* dual role. Future experiments involving the knockout of other PKC genes via the CRISPR-Cas9 system, in conjunction with enforced *prkcda* expression, may provide additional information on PKC functionality and crosstalk within the CHT.

Acknowledgements

I am deeply appreciative of Dr. Bradley Blaser's support during my tenure in his lab. His training and weekly journal club meetings improved my understanding of scientific studies pertaining to Acute Myeloid Leukemia and hematopoietic stem cell topics. I saw a great improvement in my ability to interpret scientific figures and charts following his expert mentorship in the technical aspects of research, such as flow cytometry, reverse transcription, fluorescence, electrophoresis, and polymerase chain reaction. He has undoubtedly shaped my investigative abilities in molecular genetics, which I had little knowledge of prior to his accepting me as a lab member.

Secondly, I would like to thank Dr. Mark Ziolo for his support as a second project advisor. His willingness to serve on my thesis examination committee is greatly appreciated. I would also like to thank Jami Shaffer, who provided me with many hours of training on lab procedures and always offered words of encouragement. Finally, I will not forget the professionalism of Donn Calkins, Emily Teets, and Alex Belardo.

Citations:

1. Orkin SH, Zon LI. Hematopoiesis: An evolving paradigm for stem cell biology. *Cell (Cambridge)*. 2008;132(4):631-644. doi:10.1016/j.cell.2008.01.025.
2. Perlin JR, Robertson AL, Zon LI. Efforts to enhance blood stem cell engraftment: Recent insights from zebrafish hematopoiesis. *The Journal of experimental medicine*. 2017;214(10):2817-2827. doi:10.1084/jem.20171069.
3. Blaser BW, Moore JL, Hagedorn EJ, Li B, Riquelme R. CXCR1 remodels the vascular niche to promote hematopoietic stem and progenitor cell engraftment. *The Journal of experimental medicine*. 2017;214(4):1011-1027. doi:10.1084/jem.20161616.
4. Wu TT, Hsieh YH, Wu CC, Hsieh YS, Huang CY, Liu JY. Overexpression of protein kinase Cα mRNA in human hepatocellular carcinoma: A potential marker of disease prognosis. *Clinica Chimica Acta*, 2007;*382*(1-2):54-58. doi:10.1016/j.cca.2007.03.018.
5. Palmer RH, Ridden J, Parker PJ. Cloning and expression patterns of two members of a novel protein-kinase-C-related kinase family. *Eur J Biochem*. 1995;227(1-2):344-351. doi:10.1111/j.1432-1033.1995.tb20395.x
6. Way KJ, Chou E, King G. Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends in pharmacological sciences (Regular ed.)*. 2000;21(5):181-187. doi:10.1016/s0165-6147(00)01468-1.
7. Yuan X, Chen H, Li X, et al. Inhibition of protein kinase C by isojacareubin suppresses hepatocellular carcinoma metastasis and induces apoptosis in vitro and in vivo. *Sci Rep*. 2015;5:12889. doi:10.1038/srep12889.
8. Wakasaki H, Koya D, Schoen F, Jirousek M, Ways D. Targeted overexpression of protein kinase C beta2 isoform in myocardium causes cardiomyopathy. *Proceedings of the National Academy of Sciences - PNAS*. 1997;94(17):9320-9325. doi:10.1073/pnas.94.17.9320.
9. Huang KP. The mechanism of protein kinase C activation. *Trends in neurosciences (Regular ed.)*. 1989;12(11):425-432. doi:10.1016/0166-2236(89)90091-x.
10. Cazaubon S, Webster C, Camoin L, Strosberg A, Parker P. Effector-dependent conformational changes in protein kinase C gamma through epitope mapping with inhibitory monoclonal antibodies. *European journal of biochemistry*. 1990;194(3):799-804. doi: 10.1111/j.1432-1033.1990.tb19472.x.
11. Newton AC. Protein kinase C: perfectly balanced. *Crit Rev Biochem Mol Biol.* 2018;53(2):208-230. doi:10.1080/10409238.2018.1442408.
12. Kazanietz MG, Bustelo X, Barbacid M, Kolch W, Mischak H. Zinc finger domains and phorbol ester pharmacophore. analysis of binding to mutated form of protein kinase C zeta and the vav and c-raf proto-oncogene products. *The Journal of biological chemistry*. 1994;269(15):11590-11594.
13. Osada S, Mizuno K, Saido T, Akita Y, Suzuki K. A phorbol ester receptor/protein kinase, nPKC eta, a new member of the protein kinase C family predominantly expressed in lung and skin. *The Journal of biological chemistry*. 1990;265(36):22434-22440.
14. Steinberg SF. Structural basis of protein kinase C isoform function. *Physiological reviews*. 2008;88(4):1341-1378. doi:10.1152/physrev.00034.2007.
15. Uddin S, Sassano A, Deb D, Verma A, Majchrzak B. Protein kinase C-delta (PKC-delta) is activated by type I interferons and mediates phosphorylation of Stat1 on serine 727. *The*

Journal of biological chemistry. 2002;277(17):14408-14416. doi:10.1074/jbc.M109671200.
16. Kim SJ, Chang Y, Kang S, Chun J. Phorbol ester effects in atypical protein kinase C zeta overexpressing NIH3T3 cells: Possible evidence for crosstalk between protein kinase C isoforms. Biochemical and biophysical research communications. 1997;237(2):336-339. doi:10.1006/bbrc.1997.7061.
17. Puente LG, Mireau L, Lysechko T, Ostergaard H. Phosphatidylinositol-3-kinase regulates PKCtheta activity in cytotoxic T cells. *Molecular immunology*. 2005;42(10):1177-1184. doi: 10.1016/j.molimm.2004.11.012.
18. Redig AJ, Platanias LC. Protein kinase C signaling in leukemia. *Leuk Lymphoma*. 2008;49(7):1255-1262. doi:10.1080/10428190802007726.
19. Nasser MW, Marjoram R, Brown S, Richardson R. Cross-desensitization among CXCR1, CXCR2, and CCR5: Role of protein kinase C-epsilon. *The Journal of immunology*. 2005;174(11):6927-6933. doi:10.4049/jimmunol.174.11.6927.
20. Gamal Badr G. CXC chemokine ligand 12 (CXCL12) via its cognate receptor (CXCR4) controls the chemotaxis of multiple myeloma cell line (U266) via PI3K/AKT, PLCβ3, RhoA, NFκB and ERK1/2. *African journal of pharmacy and pharmacology*. 2011;5(22):2505-2512. doi:10.5897/AJPP11.682.
21. Teicher BA, Fricker SP. CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clinical cancer research*. 2010;16(11):2927-2931. doi: 10.1158/1078-0432.CCR-09-2329.
22. Kryczek I, Wei S, Keller E, Liu R, Zou W. Stroma-derived factor (SDF-1/CXCL12) and human tumor pathogenesis. *American Journal of Physiology: Cell Physiology*. 2007;292(3):C987-C995. doi:10.1152/ajpcell.00406.2006.
23. Nervi B, Ramirez P, Rettig MP, Uy GL, Holt MS. Chemosensitization of acute myeloid leukemia (AML) following mobilization by the CXCR4 antagonist AMD3100. *Blood*. 2009;113(24):6206-6214. doi:10.1182/blood-2008-06-162123.
24. Katsura M, OHKUMA S, CHEN D, KURIYAMA K. Ethanol-induced alteration in activities of cerebral phosphatidylinositol 4,5-biphosphate-specific and cytosolic phospholipase C in the brain: Analysis using NG 108-15 cells and brains from ethanol-inhaled mice. *Neurochemistry international*. 1994;24(6):541-547. doi:10.1016/0197-0186(94)90005-1.
25. Romani A, Fulceri R, Pompella A, Ferro M, Benedetti A. Active Ca2 accumulation in the endoplasmic reticulum of different hepatomas: Stimulation by phosphates and Ca2 - releasing effect of IP 3. *Annals of the New York Academy of Sciences*. 1988:249-252. doi:10.1111/j.1749-6632.1988.tb22343.x.
26. Conesa-Zamora P. Identification of the phosphatidylserine binding site in the C2 domain that is important for PKC alpha activation and in vivo cell localization. *Biochemistry (Easton)*. 2001;40(46):13898-13905. doi:10.1021/bi011303o.
27. Ziemba BP, Li J, Landgraf KE, Knight JD, Voth GA. Single-molecule studies reveal a hidden key step in the activation mechanism of membrane-bound protein kinase C-α. *Biochemistry (Easton)*. 2014;53(10):1697-1713. doi:10.1021/bi4016082.
28. Hirano Y, Yoshinaga S, Ogura K, Yokochi M, Noda Y. Solution structure of atypical protein kinase C PB1 domain and its mode of interaction with ZIP/p62 and MEK5. *The Journal of biological chemistry*. 2004;279(30):31883-31890. doi:10.1074/jbc.M403092200.
29. Tsai LL, Xie L, Dore K, Xie L, Del Rio JC. Zeta inhibitory peptide disrupts electrostatic interactions that maintain atypical protein kinase C in its active conformation on the

scaffold p62. *The Journal of biological chemistry*. 2015;290(36):21845-21856. doi: 10.1074/jbc.M115.676221.
30. Nakagawa R, Soh J, Michie A. Subversion of protein kinase C alpha signaling in hematopoietic progenitor cells results in the generation of a B-cell chronic lymphocytic leukemia-like population in vivo. *Cancer research (Chicago, Ill.)*. 2006;66(1):527-534. doi: 10.1158/0008-5472.CAN-05-0841.
31. Blass M, Kronfeld I, Kazimirsky G, Blumberg P, Brodie C. Tyrosine phosphorylation of protein kinase cdelta is essential for its apoptotic effect in response to etoposide. *Molecular and cellular biology*. 2002;22(1):182-195. doi:10.1128/mcb.22.1.182-195.2002.
32. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. *Nature reviews*. Cancer. 2007;7(4):281-294. doi: 10.1038/nrc2110.
33. Humphries MJ, Ohm AM, Schaack J, Adwan TS, Reyland ME. Tyrosine phosphorylation regulates nuclear translocation of PKCdelta. *Oncogene*. 2008;27(21):3045-3053. doi: 10.1038/sj.onc.1210967.
34. Kajimoto T, Shirai Y, Sakai N, Yamamoto T, Matsuzaki H. Ceramide-induced apoptosis by translocation, phosphorylation, and activation of protein kinase cdelta in the golgi complex. *The Journal of biological chemistry*. 2004;279(13):12668-12676. doi: 10.1074/jbc.M312350200.
35. Zabarovsky ER, Lerman M, Minna J. Tumor suppressor genes on chromosome 3p involved in the pathogenesis of lung and other cancers. *Oncogene*. 2002;21(45):6915-6935. doi:10.1038/sj.onc.1205835.
36. Gubina E, Rinaudo M, Szallasi Z, Blumberg P, Mufson R. Overexpression of protein kinase C isoform epsilon but not delta in human interleukin-3-dependent cells suppresses apoptosis and induces bcl-2 expression. *Blood*. 1998;91(3):823-829. doi:10.1182/blood.V91.3.823.823_823_829.
37. Dhillon AS, Meikle S, Yazici Z, Eulitz M, Kolch W. Regulation of raf-1 activation and signalling by dephosphorylation. *The EMBO journal.* 2002;21(1-2):64-71. doi:10.1093/emboj/21.1.64.
38. Ding L, Wang H, Lang W, Xiao L. Protein kinase C-epsilon promotes survival of lung cancer cells by suppressing apoptosis through dysregulation of the mitochondrial caspase pathway. *The Journal of biological chemistry*. 2002;277(38):35305-35313. doi:10.1074/jbc.M201460200.
39. Pan Q, Bao L, Kleer C, Sabel M, Griffith K. Protein kinase C epsilon is a predictive biomarker of aggressive breast cancer and a validated target for RNA interference anticancer therapy. *Cancer research (Chicago, Ill.)*. 2005;65(18):8366-8371. doi:10.1158/0008-5472.CAN-05-0553.
40. Cornford P, Evans J, Dodson A, Parsons K, Woolfenden A. Protein kinase C isoenzyme patterns characteristically modulated in early prostate cancer. *The American journal of pathology*. 1999;154(1):137-144. doi:10.1016/S0002-9440(10)65260-1.
41. Garg R, Benedetti LG, Abera MB, Wang H, Abba M. Protein kinase C and cancer: What we know and what we do not. *Oncogene*. 2014;33(45):5225-5237. doi:10.1038/onc.2013.524.
42. Reina-Campos M, Diaz-Meco MT, Moscat J. The dual roles of the atypical Protein Kinase Cs in cancer. *Cancer cell.* 2019;36(3):218-235. doi:10.1016/j.ccell.2019.07.010.

43. Ali SA, Justilien V, Jamieson L, Murray NR, Fields AP. Protein kinase Cι drives a NOTCH3-dependent stem-like phenotype in mutant KRAS lung adenocarcinoma. *Cancer cell*. 2016;29(3):367-378. doi:10.1016/j.ccell.2016.02.012.
44. Justilien V, Ali SA, Jamieson L, Yin N, Cox AD. Ect2-dependent rRNA synthesis is required for KRAS-TRP53-driven lung adenocarcinoma. *Cancer cell*. 2017;31(2):256-269. doi: 10.1016/j.ccell.2016.12.010.
45. Haug MF, Gesemann M, Berger M, Neuhauss SCF. Phylogeny and distribution of protein kinase C variants in the zebrafish. *J Comp Neurol.* 2018;526(7):1097-1109. doi:10.1002/cne.24395
46. Jami L Shaffer, Donn L Calkins, Emily M Teets, Muhammad Faisal, William Deruelle, Elliott J. Hagedorn, Julie R. Perlin, Serine Avagyan, Leonard I. Zon, Bradley W Blaser; Cxcl8/PKC Signaling in the Endothelial Cell Niche Regulates Long-Term Hematopoietic Stem Cell Fate. *Blood* 2020; 136 (Supplement 1): 38. doi:10.1182/blood-2020-141609
47. Araki S, Omori Y, Lyn D, Singh RK, Meinbach DM. Interleukin-8 is a molecular determinant of androgen independence and progression in prostate cancer. *Cancer research (Chicago, Ill.)*. 2007;67(14):6854-6862. doi:10.1158/0008-5472.CAN-07-1162.
48. Grossoni VC, Falbo KB, Kazanietz MG, Joffe, Elisa D. Bal de Kier, Urtreger AJ. Protein kinase C delta enhances proliferation and survival of murine mammary cells. *Molecular carcinogenesis*. 2007;46(5):381-390. doi:10.1002/mc.20287.